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Similarity and Divergence among Viruses in the Genus *Furovirus*[1]

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Nucleotide sequences of RNAs 1 and 2 of a Japanese strain of soil-borne wheat mosaic virus (SBWMV), the type species of the genus *Furovirus,* and sorghum chlorotic spot virus (SCSV) were determined from cloned cDNA. The relationship among the Japanese and US strains of SBWMV, SCSV, oat golden stripe virus (OGSV), and recently proposed Chinese wheat mosaic and European wheat mosaic viruses (CWMV and EWMV) were examined at the nucleotide and amino acid levels. Pairwise comparisons of genome-encoded proteins among the six viruses showed that the US strains of SBWMV and CWMV were the most closely related pair in RNA 1 and the Japanese strains of SBWMV and EWMV were most closely related in RNA 2. SCSV was most distantly related to the other five viruses. Phylogenetic analysis indicated that there may have been an ancient reassortment between RNAs 1 and 2 of the four wheat-infecting viruses and OGSV, while SCSV was shown to have separated from the rest before the other five viruses diverged. The fact that CWMV and EWMV have almost identical biological properties as well as the sequence similarities to the two strains of SBWMV suggests that they be regarded as strains of SBWMV, considering that SBWMV consists of genetically diverged strains. OGSV and SCSV are distinct in biological properties in addition to genetic divergence in the genus *Furovirus.*

INTRODUCTION

The genus *Furovirus* consists of rigid rod-shaped viruses with bipartite positive-sense RNA genomes which infect gramineous plants and are transmitted by *Polymyxa graminis* in soil (Shirako and Wilson, 1994). Historically the "furovirus group" included other rod-shaped multipartite viruses with fungal vectors (Shirako and Brakke, 1984a; Brunt and Richards, 1989) but accumulation of information on genome structures of many virus species in this category enabled Torrance and Mayo (1997) to clarify ambiguous relationships among the rod-shaped fungus-transmitted viruses. Thus the previous furovirus group was reclassified into four genera, *Furovirus* with soil-borne wheat mosaic virus (SBWMV) as the type species, *Pomovirus* with potato mop-top virus as the type species, *Pecluvirus* with peanut clump virus as the type species, and *Benyvirus* with beet necrotic yellow vein virus as the type species (Mayo, 1999). Each genus is differentiated based on the number of viral RNA segments, types of proteins required for cell-to-cell movement, nucleotide sequence structures at the 3′-terminal region, and distant phylogenetic relationships based on amino acid sequences of the encoded proteins (Torrance and Mayo, 1997).

Recently nucleotide sequences of RNAs 1 and 2 of viruses infecting wheat plants in China (Chen, 1993) and France and oat golden stripe virus (OGSV) were published (Diao *et al.,* 1999a,b). The former two wheat-infecting viruses had been considered strains of SBWMV but the authors concluded that they are distinct species, Chinese wheat mosaic virus (CWMV) and European wheat mosaic virus (EWMV), in the genus *Furovirus,* based on sequence divergence at the nucleotide and amino acid levels from the Nebraska isolate of SBWMV (Shirako and Wilson, 1993).

Here we report the nucleotide sequences of RNAs 1 and 2 of a Japanese strain of SBWMV (Shirako and Ehara, 1986) and sorghum chlorotic spot virus (SCSV) (Kendall *et al.,* 1988) and compare them with those of other rod-shaped plant viruses including pomo-, peclu-, hordei-, tobra-, tobamo-, and benyviruses. Our results indicate that the Japanese and US strains of SBWMV, CWMV, EWMV, and OGSV are closely related, based on amino acid sequence identities of the encoded proteins; that SCSV is more distantly related to the other viruses in the genus *Furovirus;* and that CWMV and EWMV may be considered as strains of SBWMV, based on the nearly identical biological properties as well as the sequence similarities.

[1] The nucleotide sequence data reported in this paper have been submitted to DDBJ and assigned Accession Nos. AB033689 (SBWMV-JT RNA 1), AB033690 (SBWMV-JT RNA 2), AB033691 (SCSV RNA 1), and AB033692 (SCSV RNA 2).

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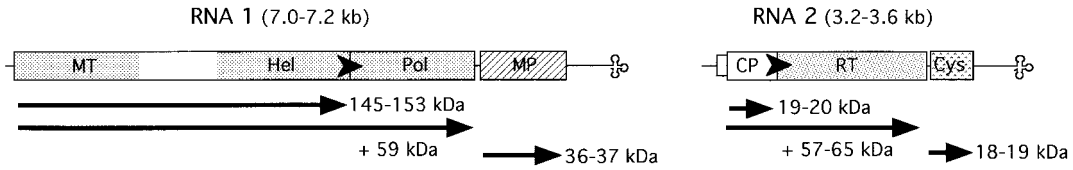

FIG. 1. Genome organization of RNA 1 and RNA 2 of the genus *Furovirus*. MT, putative methyltransferase domain; Hel, putative helicase domain; Pol, putative RNA polymerase domain; MP, putative cell-to-cell movement proteins; CP, the capsid proteins; RT, capsid/readthrough region; Cys, cysteine-rich protein. Wedge-shaped arrows between Hel and Pol and between CP and RT indicate partial readthrough at the leaky opal termination codon at the ends of the MT-Hel gene and the CP gene. Clover-shaped figures at the 3′ termini of RNA 1 and RNA 2 indicate a tRNA-like structure.

RESULTS

Genome structure of a Japanese strain of SBWMV and SCSV

The Japanese strain of SBWMV and SCSV had the same genome organization as the US strain of SBWMV (Shirako and Wilson, 1993) (Fig. 1).

The sizes of RNAs 1 and 2 of the Japanese strain of SBWMV were 7225 and 3574 nucleotides, respectively. Between the Japanese and Nebraskan strains of SBWMV, the highest amino acid sequence identity was found in the capsid protein with 82%, which was higher than that in the RNA polymerase domain with 78%, and the lowest was in the readthrough region in RNA 2 with 62%.

Nucleotide sequence analysis of SCSV RNAs 1 and 2 could not be completed as a result of the failure to determine the 5′-terminal nucleotide sequences. Primer extension experiments showed that about 30 nucleotides in RNA 1 and about 150 nucleotides in RNA 2 toward the 5′ terminus were not determined by reason of nonspecific terminations probably caused by partial degradation of RNA from the 5′ terminus during a purification procedure or by strong RNA secondary structures which may have caused reverse transcriptase to fall off under the standard primer extension reaction condition. Including the numbers of sequence ladders formed after the primer extension reactions, the sizes of RNAs 1 and 2 of SCSV were about 6900 and 3600 nucleotides, respectively. Between SCSV and the US strain of SBWMV, the highest amino acid sequence identity was found in the RNA polymerase domain with 72%, whereas the capsid protein had only 49% identity, and the lowest amino acid sequence identity was found in the "cysteine-rich" protein encoded in the 3′-terminal region of RNA 2 with only 36%.

Comparison among six furoviruses at amino acid levels

Table 1 shows pairwise comparisons of all six proteins and domains encoded on RNAs 1 and 2 among six furoviruses.

Among RNA 1-encoded proteins, the RNA polymerase domain with 59 kDa, which is located downstream of the leaky opal termination codon for the N-terminal, putative methyltransferase-helicase protein with 145–153 kDa, was most highly conserved. The US strains of SBWMV and CWMV are most closely related in this domain with 90% identity. In this domain, SCSV was most distantly related to the other five furoviruses with an average identity of 74%. In the N-terminal, putative methyltransferase-helicase protein, the US strains of SBWMV and CWMV were most closely related with 89% identity, whereas SCSV was most distantly related to the others with an average identity of 62%. In the case of the 37-kDa putative cell-to-cell movement protein, again the US isolates of SBWMV and CWMV were most closely related with 76% identity. SCSV had an average identity of only 42% to the other furoviruses in this protein.

Among RNA 2-encoded proteins, the Japanese strains of SBWMV and EWMV were the most closely related pair in all three proteins among the six furoviruses: 92% in the capsid protein; 74% in the region C-terminally adjacent to the capsid protein, which is expressed by readthrough of the leaky opal termination codon for the capsid protein gene; and 83% in the 3′-terminal cysteine-rich protein. On the other hand, SCSV was most distantly related in all three proteins among the furoviruses with the average identities of 49% in the capsid protein, 39% in the readthrough region, and 36% in the cysteine-rich proteins.

Phylogenetic analyses of the RNA polymerase domain and the capsid protein, including representative members of all rod-shaped plant RNA viruses, showed the relationship of furoviruses with viruses in other genera (Fig. 2). Clearly, the six furoviruses formed a single cluster in both trees. Analysis by the maximum likelihood and quartet puzzling approach (Strimmer and von Haeseler, 1996) gave essentially the same tree topologies (data not shown) with the SCSV branches basal to the other furoviruses in both the capsid protein and polymerase domain trees. The relative affinities among the five other furoviruses were not congruent between the two trees, however, with the Japanese strain of SBWMV and OGSV clustering separately in the polymerase domain tree, while the two strains of SBWMV and EWMV formed a distinct cluster in the capsid protein tree (Fig. 2). The order of similarities using the polymerase domain was, from highest to lowest, between *Furovirus* and *Pomovi-*

TABLE 1

Pairwise Comparisons of Proteins and Domains Encoded on RNA 1 and RNA 2 of Six Furoviruses

	Pol (RNA 1)					
	SBWMV-NE	SBWMV-JT	CWMV	EWMV	OGSV	SCSV
MT/Hel (RNA 1)						
SBWMV-NE	—	78	**90**	82	78	72
SBWMV-JT	70	—	78	81	87	77
CWMV	**89**	68	—	82	77	72
EWMV	83	69	83	—	79	73
OGSV	70	79	70	72	—	77
SCSV	58	62	69	59	61	—
	RT (RNA 2)					
CP (RNA 2)						
SBWMV-NE	—	62	58	59	51	39
SBWMV-JT	82	—	53	**74**	52	40
CWMV	75	69	—	52	49	39
EWMV	79	**92**	71	—	49	38
OGSV	77	73	77	71	—	38
SCSV	49	49	50	49	47	—
	37K (RNA 1)					
Cys-rich (RNA 2)						
SBWMV-NE	—	63	**76**	69	58	41
SBWMV-JT	66	—	62	62	56	40
CWMV	61	61	—	64	59	42
EWMV	62	**83**	61	—	56	43
OGSV	63	67	66	63	—	42
SCSV	36	36	37	37	36	—

Note. Designations of each protein are as defined in Fig. 1. In each region, the highest score was boxed. In MT/Hel, the N-terminal 560-amino acid and the C-terminal 460-amino acid sequences were combined and analyzed.

rus, followed by *Pecluvirus, Hordeivirus, Tobravirus, Tobamovirus,* and *Benyvirus.* Using the capsid protein in the analysis, *Furovirus* and *Pomovirus* formed one cluster, while peclu-, hordei-, tobra-, and tobamoviruses formed another cluster. The genus *Benyvirus* was most distantly related to the rest of the genera among all rod-shaped plant RNA viruses.

The 3′-terminal nucleotide sequence

Figure 3 shows aligned 100-nucleotide sequences in the 3′-terminal region of RNAs 1 and 2 of six furoviruses, except for that of RNA 2 of OGSV. In all nucleotide sequences, it was shown that the 3′-terminal 82-nucleotide region may form tRNA-like structures containing four stem-loops. There was a putative valine anticodon in the third loop region from the 3′ terminus. A nucleotide change in one strand of a stem caused a complementary change in the other strand to form a base pair. Nucleotide variations among 11 nucleotide sequences occurred more frequently outside of the sequence-forming stems (1.4%) rather than those in stems (0.8%), indicating that formation of four stem-loop structures, which may be folded into a tRNA-like structure, is significant. Indeed, it has been experimentally shown that the 3′ terminus of a US strain of SBWMV RNA could be valinated *in vitro* (Goodwin and Dreher, 1998).

Comparison of the capsid readthrough region of the Japanese strain of SBWMV and SCSV

The capsid readthrough region of the Japanese strain of SBWMV and SCSV were analyzed for their hydrophobicity profile using the method of Kyte and Doolittle (1982) (Fig. 4A) and by dot-matrix comparison with the window size 15 and stringency 6 (Fig. 4B). Despite their distant relationship with an amino acid sequence identity of only 40%, the two proteins shared a similar hydrophobicity profile containing two hydrophobic peaks: one between the amino acid positions 106 and 129 (position 1 is the first amino acid after the leaky opal termination codon at the end of the capsid protein) in the Japanese strain of SBWMV and between the positions 95 and 123 in SCSV, and another between positions 372 and 393 in Japanese SBWMV and between 347 and 369 in SCSV. Dot-matrix comparison showed that the C-terminal 160-amino acid region is not conserved well between the two

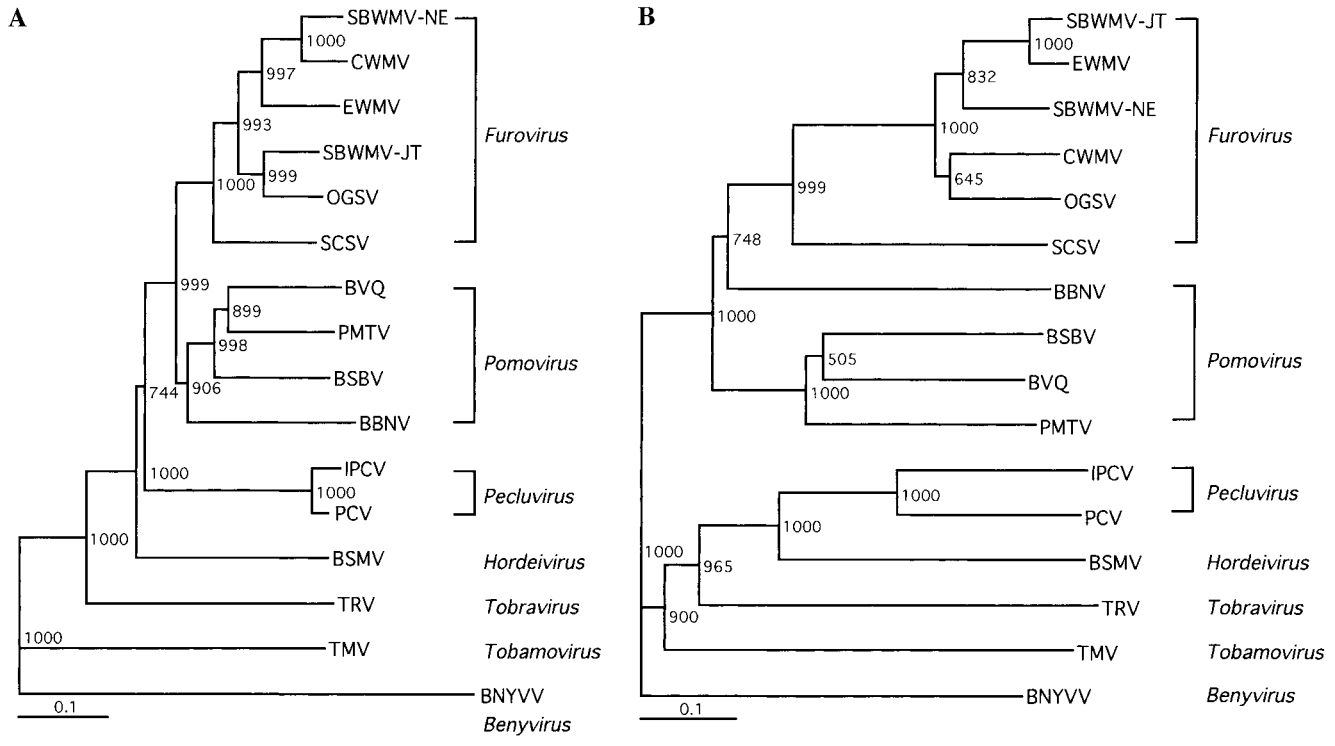

FIG. 2. Phylogenetic analysis of rod-shaped plant RNA viruses using RNA polymerase domain (A) and the capsid protein (B). Phylogenetic trees were obtained using the neighbor joining method of ClustalX (Thompson *et al.,* 1997) and displayed as phylograms in the program TREEVIEW. The bootstrap values for 1000 replicates are shown above each branch. The bars at the bottom represent distances scaled as substitutions per amino acid residue. Virus names and accession numbers for (A and B) are as follows. SBWMV-NE (US strain, Nebraska isolate), L07937 and L07938; SBWMV-JT (Japan strain, JT isolate), AB033689 and AB033690; CWMV, AJ012005 and AJ012006; EWMV, AJ132576 and AJ132577; OGSV, AJ132578 and AJ132579; SCSV, AB033691 and AB033692; BVQ (beet virus Q), AJ223596 and AJ223597; PMTV (potato mop-top virus), AJ238607 and D16193; BSBV (beet soil-borne virus), Z97873 and U64512; BBNV (broad bean necrosis virus), D86636 and D86637; IPCV (Indian peanut clump virus), X99149 and X76658; PCV (peanut clump virus), X78602 and L07269; BSMV (barley stripe mosaic virus), M16576 (C-terminal 506 amino acids) and X03854; TRV (tobacco rattle virus), X06172 and X03686; TMV (tobacco mosaic virus), J02415; BNYVV (beet necrotic yellow vein virus), X05147 (C-terminal 506 amino acids) and X04197.

proteins (Fig. 4B). Compared with the readthrough region of the four wheat-infecting furoviruses, SCSV has a deletion of about 30 amino acids in the N-terminal region (as shown by an arrow in Fig. 4B), where spontaneous deletion usually does not occur (Chen *et al.,* 1995; Nishimura and Shirako, unpublished observations).

The capsid readthrough region of the US strain of SBWMV, CWMV, EWMV, and OGSV shared the same characteristics with that of the Japanese strain of SBWMV compared with SCSV in terms of their hydrophobicity profiles and dot-matrix patterns. Only the readthrough region of OGSV was shorter than others in the C-terminal region as previously shown (Diao *et al.,* 1999a).

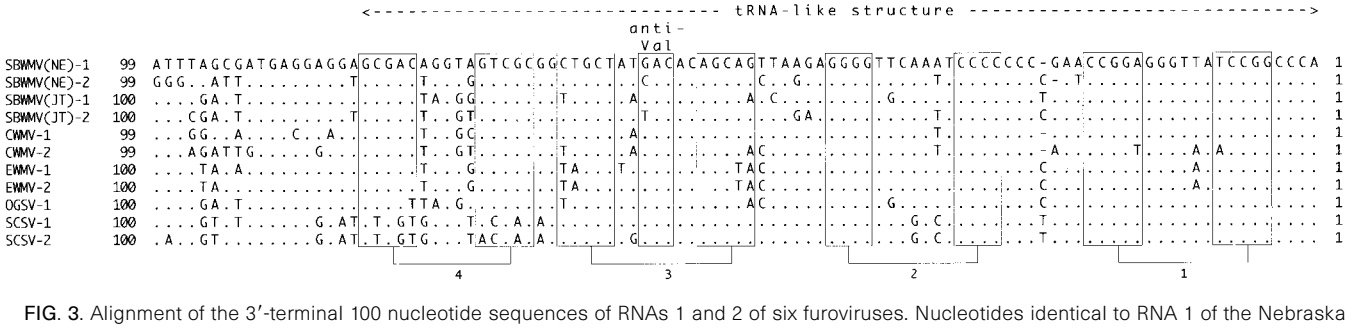

FIG. 3. Alignment of the 3′-terminal 100 nucleotide sequences of RNAs 1 and 2 of six furoviruses. Nucleotides identical to RNA 1 of the Nebraska isolate of SBWMV are indicated by dots. Dashes were inserted to maximize alignment. Nucleotide sequences possibly forming stem structures are boxed and connected by lines below the boxes with the stems numbered from the 3′ end. A possible valine anticodon in the loop above the stem 3 was also boxed. Nucleotide numbers are from the 3′ terminus.

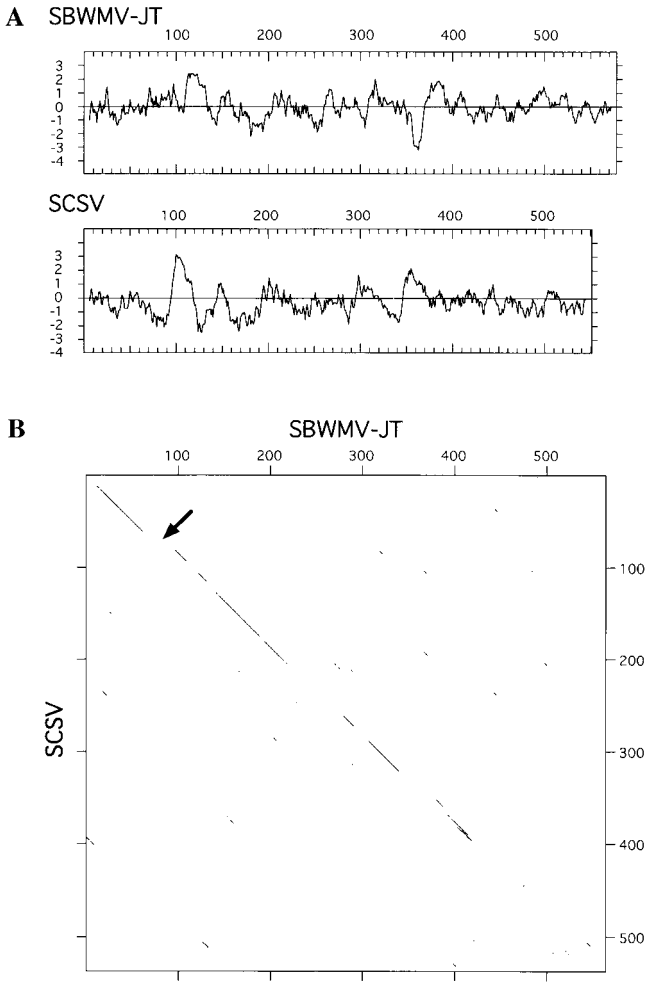

FIG. 4. Comparison of the capsid/readthrough region of the Japanese strain of SBWMV and SCSV. (A) Hydrophobicity profiles by the method of Kyte and Doolittle (1982). (B) Dot-matrix comparison with the window size of 15 and the stringency of 6. An arrow indicates a region with about 30 amino acids, which is deleted in SCSV relative to all other viruses in the genus *Furovirus.*

DISCUSSION

The previous furovirus (fungus-transmitted rod-shaped) group (Shirako and Brakke, 1984a; Shirako and Wilson, 1994) has been reclassified recently into four genera, *Furovirus, Pomovirus, Pecluvirus,* and *Benyvirus,* based on the different genome structure and organization revealed by complete nucleotide sequence information of most of the members of the previous furovirus group (Torrance and Mayo, 1997; Mayo, 1999). The genus *Furovirus* now consists of SBWMV (Brakke, 1971), OGSV (Adams *et al.,* 1988), SCSV (Kendall *et al.,* 1988), and recently proposed CWMV and EWMV (Diao *et al.,* 1999a,b), although other new members may be discovered in the future.

In this study we reconsidered the *Furovirus* classification by comparing the newly determined nucleotide sequences of a Japanese strain of SBWMV and SCSV with those of the US strain of SBWMV (Shirako and Wilson, 1993), CWMV, EWMV, and OGSV (Diao *et al.,* 1999a,b). Our analysis indicated that there is substantial divergence among wheat-infecting furoviruses at the genomic level despite their near identity in biological properties (Table 2). The average amino acid identity from pairwise comparisons among the two strains of SBWMV and proposed CWMV and EWMV was 82% in the RNA polymerase domain and 78% in the capsid protein. That the US strains of SBWMV and CWMV are most closely related in RNA 1, whereas the Japanese strain of SBWMV and EWMV are most closely related in RNA 2 among the six viruses, suggests that there may have been a reassortment between two genomic RNA segments among the four wheat-infecting viruses during the course of evolution. Another possibility for nonparallel divergence of RNA 1 and RNA 2 is that the four wheat-infecting furoviruses and OGSV underwent sequential periods of divergent and convergent evolution after the initial split from the SCSV lineage. In either case, a close biological relationship among the four wheat-infecting viruses is obvious (Table 2). They infect the same hosts, cause similar diseases, are probably all transmitted in soil by *Polymyxa graminis,* and require a low temperature for infection. Considering these biologically common properties as well as the sequence

TABLE 2

Comparison of Biological Properties among Viruses in the Genus *Furovirus*

	SBWMV-US[a]	SBWMV-Japan[b]	CWMV[c]	EWMV[d]	OGSV[e]	SCSV[f]
Transmission	*P. graminis*	*P. graminis*	in soil	in soil	*P. graminis*	unknown
Host plants	wheat/barley	wheat/barley	wheat	wheat	oat	sorghum
Diseases	mosaic/stunt	mosaic/stunt	mosaic/stunt	mosaic/stunt	mosaic/stunt	yellow stripe
Optimal growth temperature	17°C	17°C	~17°C	~17°C	15°C	25°C

[a] Brakke, 1977.
[b] Tsuchizaki *et al.,* 1973; Shirako and Ehara, 1986.
[c] Chen, 1993; Diao *et al.,* 1999b.
[d] Diao *et al.,* 1999a.
[e] Plumb *et al.,* 1977; Adams *et al.,* 1988; Diao *et al.,* 1999a.
[f] Kendall *et al.,* 1988.

similarities among the four viruses, CWMV and EWMV can be regarded as strains of SBWMV as in the case of the Japanese and US strains of SBWMV. One could argue that the Japanese SBWMV strain is a *Furovirus* species different from the US strain of SBWMV based only on the sequence divergence found between the two viruses, just in the case for CWMV and EWMV. However, this would be inappropriate given the interchangeability of genomic RNAs between the two SBWMV strains along with the identical biological characteristics. Tsuchizaki *et al.* (1973, 1975) reported that it is possible to generate pseudorecombinant viruses between Japanese and US strains of SBWMV. Their results clearly demonstrate that, although the two strains are not the closest pair either in RNA 1 or in RNA 2 among the examined furoviruses, corresponding proteins of the two strains functionally complement each other. It will be worthwhile to examine compatibility of RNAs 1 and 2 of CWMV and EWMV with those of the two strains of SBWMV to draw a firm classification of the four wheat-infecting furoviruses. SBWMV can be considered to consist of genetically diverged strains.

OGSV should be a separate species in the genus *Furovirus,* since it does not infect either wheat or barley by mechanical inoculation (Plumb *et al.,* 1977), indicating the host range is determined by replication and/or movement in inoculated plants but probably not by vector specificity. It is noteworthy that the 37-kDa movement protein of OGSV is distantly related to those of wheat-infecting furoviruses (57% average identity) compared with the movement protein of wheat-infecting furoviruses relating among themselves (66% average identity).

Among the furoviruses, differences of SCSV from the rest at the genomic level and in biological properties are significant. SCSV is known to occur only in a small area in the Midwest region of the United States and was restricted to a certain variety of sorghum (Kendall *et al.,* 1988). Although SCSV has not yet been shown to be transmitted in soil, RNA 2 codes for the readthrough protein, which is hypothesized to be required for fungus transmission (Shirako and Brakke, 1984b). In the case of SBWMV, the gene was frequently deleted internally when the virus was maintained for long periods or repeatedly passaged in wheat plants in a greenhouse (Shirako and Brakke, 1984b; Chen *et al.,* 1994). SCSV has a similar hydrophobicity profile including two large hydrophobic peaks which are proposed to interact within a membrane (Diao *et al.,* 1999a). Further study is required to determine whether SCSV is fungal-transmissible, or whether the readthrough region is required for fungus transmission in *Furovirus,* as it is in the genera *Pomovirus* (Reavy *et al.,* 1998) and *Benyvirus* (Tamada and Kusume, 1991; Tamada *et al.,* 1996).

MATERIALS AND METHODS

Viruses and purification

A Japanese strain of SBWMV (isolate JT) was propagated in wheat plants (*Triticum aestivum,* cv. Fukuho) and purified as described previously (Shirako and Ehara, 1986). SCSV (Kendall *et al.,* 1988) was maintained in maize (*Zea mays,* inbred line N28Ht) and purified using the method of Shirako and Brakke (1984a).

cDNA cloning and determination of nucleotide sequence

RNA 1 and RNA 2 of SBWMV were purified by sucrose density-gradient centrifugation, followed by ethanol precipitation as described before (Shirako and Ehara, 1986). SCSV RNA was extracted in bulk from purified virus by an SDS/phenol method and precipitated in ethanol. To extracted RNA, cDNA was transcribed with a random hexadeoxyoligonucleotide primer using AMV reverse transcriptase (Life Sciences). The second-strand synthesis was done by the method of Gubler and Hoffman (1983), followed by treatment with T4 DNA polymerase in the presence of RNase A. After methylation of an internal *Eco*RI site, ds cDNA was ligated with an *Eco*RI linker and cloned into *Eco*RI-digested pGEM3Z (Promega). Ligation products were transformed into *Escherichia coli* MC1061 cells. After recombinant plasmids were screened for the presence of inserts longer than 1.0 kb, the nucleotide sequences of the inserts were determined from both ends using SP6 and T7 primers with an ABI377 automated sequencer (ABI) or manually using [α-^{32}P]dATP by the dideoxy method. For the 3′-terminal sequence determination, the terminus was polyadenylated with *E. coli* poly (A) polymerase (Pharmacia) and cDNA was synthesized with a d(T)17 primer, followed by cloning of ds cDNA into a plasmid for sequencing. The 5′-terminal nucleotide sequence was determined from cloned DNA after RT-PCR of a G-tailed cDNA or by a direct primer extension method using extracted viral RNA.

Computer analysis of amino acid sequences

Pairwise comparisons of translated amino acid sequences were done by the DNASIS software (Hitachi). Phylogenetic analysis was performed using the computer program ClustalX (Thompson *et al.,* 1997). Sequences were aligned using the Gonnet series weight matrix, a gap weight of 10.0, and gap length weight of 0.2. A distance matrix was calculated from the alignment and phylogenetic trees were constructed by the neighbor-joining method with 1000 bootstrap replicates. Phylogenetic trees were also reconstructed using a maximum likelihood method and quartet puzzling as implemented in the computer program Puzzle 4.02 (Strimmer and von Haeseler, 1996) using the JTT model. Trees were displayed using the program TREEVIEW (Page, 1996). Hy-

drophobicity analysis and a dot-matrix comparison were done using the DNA Strider software (Marck, 1988; Kyte and Doolittle, 1982).

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Note added in proof. After submission of this article R. Koenig *et al.* published a paper reporting a furovirus from rye and wheat, which is nearly identical to the EWM strain of SBWMV (1999, *Arch. Virol.* **144,** 2125–2140).

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